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<b>(21) International Application Number:</b> PCT/US89/00931 <b>(22) International Filing Date:</b> 8 March 1989 (08.03.89) <b>(31) Priority Application Number:</b> 169,949 <b>(32) Priority Date:</b> 18 March 1988 (18.03.88) <b>(33) Priority Country:</b> US  <b>(71) Applicant:</b> THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, U.S. DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road, Springfield, VA 22161 (US).  <b>(72) Inventors:</b> MOSS, Bernard ; 10301 Dickens Avenue, Bethesda, MD 20814 (US). FALKNER, Falko, G. ; A-2304 Mannsdorf 116 (AT).	<b>(74) Agents:</b> STERN, Marvin, R. et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, 400 Seventh Street, N.W., Washington, DC 20004 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> NOVEL RECOMBINANT VACCINIA VIRUS EXPRESSION VECTORS AND METHOD OF SELECTING SAME  <b>(57) Abstract</b>  Recombinant plasmids and vaccinia virus expression vectors which allow dominant selection have been made.		

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NOVEL RECOMBINANT VACCINIA VIRUS EXPRESSION  
VECTORS AND METHOD OF SELECTING SAME

TECHNICAL FIELD

The present invention is related generally to construction of recombinant vaccinia virus vectors. More particularly, the present invention is related to the construction of unique vaccinia virus open-reading-frame expression vectors and a method for dominant selection of the same.

BACKGROUND OF THE INVENTION

Vaccinia virus is a useful vector for gene expression in mammalian cells. Advantages include the maintenance of infectivity, wide host range, large DNA capacity and correct synthesis, processing and transport of proteins. Because transcription of vaccinia virus genes is carried out by virus encoded enzymes in the cytoplasm and splicing of RNA does not occur, there are requirements for vaccinia promoters and uninterrupted open-reading-frames. In addition, the large size and lack of infectivity of the vaccinia virus genome prohibits the construction of recombinants by standard in vitro cloning techniques.

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1           A two-step procedure has been developed to  
2 overcome these difficulties. In the first step, a  
3 plasmid is constructed that contains foreign gene(s)  
4 controlled by vaccinia promoter(s), flanked by sequences  
5 derived from a non-essential site on the vaccinia  
6 genome. In the second step, the foreign genetic material  
7 in the plasmid vector is inserted into the viral genome  
8 by homologous recombination in vivo.

9           However, selection or isolation of the recombinant  
10 virus thus produced, is not easily accomplished by the  
11 presently known techniques such as plaque hybridization,  
12 thymidine kinase (tk) negative selection,  $\beta$ -galactosidase  
13 expression and the like.

14          Of these, only insertional inactivation of the tk  
15 gene is a true selection step. Disadvantages of this  
16 method, however, include requirements for: inactivation  
17 of the viral tk gene which attenuates virus infectivity,  
18 use of special tk cell lines, and use of mutagenic  
19 selective agents, such as 5-bromodeoxyuridine. In  
20 addition, spontaneous tk mutants arise at a high  
21 frequency, necessitating additional steps to distinguish  
22 them from the recombinants.

#### 23                                   SUMMARY OF THE INVENTION

24           It is, therefore, an object of the present  
25 invention to provide a new recombinant vaccinia virus  
26 expression vector allowing dominant selection of the  
27 recombinant.

28           It is a further object of the present invention to  
29 provide a vaccinia virus recombinant expression vector  
30 the genome of which includes E. coli gpt gene allowing  
31 formation of the recombinant vaccinia plaques on a  
32 plurality of cell lines when replicated in a growth

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1 medium comprising mycophenolic acid (MPA) and a substrate  
2 for purine metabolism.

3 It is another object of the present invention to  
4 provide a novel method for selecting recombinant vaccinia  
5 virus expression vectors containing a foreign gene which  
6 is desired to be expressed by said recombinant vaccinia  
7 virus.

8 Other objects and advantages of the present  
9 invention will become evident from the following detailed  
10 description of the invention.

#### 11 BRIEF DESCRIPTION OF THE DRAWINGS

12 These and other objects, features and many of the  
13 attendant advantages of the invention will be better  
14 understood upon a reading of the following detailed  
15 description when considered in connection with the  
16 accompanying drawings wherein:

17 Fig. 1 demonstrates plaque formation of vaccinia  
18 virus in the presence and absence of MPA. Confluent BSCI  
19 cells were preincubated overnight (12-16 hours) in  
20 selective medium and subsequently infected with 1,000 PFU  
21 of vaccinia wild-type virus (A, B) or a recombinant virus  
22 that expresses the E. coli gpt gene (C, D). The cells  
23 were incubated for two days in the presence (A, C) or in  
24 the absence (B, D) of MPA, xanthine, and hypoxanthine and  
25 then stained with crystal violet.

26 Fig. 2 shows the results of genomic analysis of  
27 viruses from six randomly picked gpt plaques. Southern  
28 blots were prepared from HindIII digested DNA. On the  
29 right side, the fragment sizes (in kbp) of a phage lambda  
30 HindIII digest are indicated by arrows. (A) The Southern  
31 blot was hybridized with a [<sup>32</sup>P]dCTP labeled gpt gene  
32 specific probe. Lanes 1-6, DNA of BSCI cells infected

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1 with virus clones No. 1-6; lane 7, DNA of uninfected BSCI  
2 cells; lanes 8 and 9 have 10 and 100 ng of vaccinia  
3 wild-type DNA, respectively. (B) The same Southern blot  
4 hybridized with a vaccinia virus tk gene specific probe  
5 is shown. In lanes 8 and 9, the vaccinia virus 5.1 kbp  
6 HindIII-J fragment is visible; this fragment contains the  
7 tk gene.

8 Fig. 3 shows schematic construction of the  
9 vectors. DNA sequences including the vaccinia tk gene,  
10 E. coli gpt gene, and plasmid vector are indicated by  
11 filled, empty, and cross-hatched regions on the diagrams,  
12 respectively. Arrows show the direction of transcription  
13 from the 11 K (P11), the 7.5 K (P7.5), and tk gene  
14 promoters. The pUC sequences contain the ampicillin  
15 resistance gene and the origin of bacterial replication.  
16 The unique cloning sites downstream of the 11 K gene  
17 initiation codon are indicated.

18 Fig. 4 shows the sequence of the multiple cloning  
19 sites downstream of the 11 K gene initiation codon. The  
20 frameshift mutations were included by inserting  
21 additional G residues (arrows) downstream of the ATG  
22 codon. Note that only the restriction sites EcoRI, SalI,  
23 HincII, AccI, BamHI, and HpaI are unique. The initiation  
24 and the termination codons are boxed.

25 Fig. 5 shows the results of  $\beta$ -galactosidase  
26 expression in cells infected with the recombinant  
27 viruses. Confluent CVI cells ( $2.5 \times 10^6$ ) were infected  
28 with 7.5 PFU/cell of the indicated virus; after about 24  
29 hour incubation, cytoplasmic extracts were prepared and  
30 the protein content and specific  $\beta$ -galactosidase activity  
31 were determined. The virus vFlsB was derived from the  
32 vector pTKgpt-Fls; the virus vFlsB is a derivative of  
33 pTKgpt-oFls; and vtat was derived from pSC11. T7 refers  
34 to the viruses vTF7-3

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1 (this virus expresses the bacteriophage T7 RNA  
2 polymerase) and vTFgal2 (a virus expressing the lacZ  
3 behind the T7 promoter); cells were infected with 7.5  
4 PFU/cell of each of the two viruses. Prior to infection,  
5 the titers of all viruses were re-determined.  
6  $\beta$ -galactosidase activities are mean value of two  
7 independent experiments.

8

DETAILED DESCRIPTION OF THE INVENTION

9

10 The above and various other objects and advantages  
11 of the present invention are achieved by a method for  
12 dominant selection including a vaccinia recombinant  
13 expression vector comprising in genome thereof an E. coli  
14 gpt gene and one or more foreign genes desired to be  
15 expressed by the recombinant virus, said recombinant  
16 virus forming plaques on a plurality of infectable cell  
17 lines when replicated in a growth medium comprising  
18 sufficient amount of mycophenolic acid to inhibit purine  
19 metabolism in the presence of sufficient amount of an  
unphosphorylated purine substrate.

20 The vectors of the present invention include in  
21 the genome a promoter that provides high level of  
22 expression and may include translation initiation and  
23 termination codons, and multiple restriction sites in  
24 three different frames which permit expression of partial  
25 or complete foreign genes.

26 Unless defined otherwise, all technical and  
27 scientific terms used herein have the same meaning as  
28 commonly understood by one of ordinary skill in the art  
29 to which this invention belongs. Although any methods  
30 and materials similar or equivalent to those described  
31 herein can be used in the practice or testing of the  
32 present invention, the preferred methods and materials

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1 are now described. All publications mentioned hereunder  
2 are incorporated herein by reference. Unless mentioned  
3 otherwise, the techniques employed herein are standard  
4 methodologies well known to one of ordinary skill in the  
5 art.

## 6 MATERIALS AND METHODS

7 Enzymes and chemicals. Restriction endonucleases  
8 and low melting agarose were obtained from Bethesda  
9 Research Laboratories. T4 polymerase was from  
10 Pharmacia. Enzymes were used according to the  
11 instructions of the suppliers. MPA was from CalBiochem.  
12 Xanthine and hypoxanthine were from Sigma Chemicals. MPA  
13 and xanthine were dissolved in 0.1 N NaOH, hypoxanthine  
14 was dissolved in water and sterile filtered; the  
15 solutions were stored frozen as 10 mg/ml stocks.

16 Virus and cells. Vaccinia virus (strain WR) was  
17 originally from the American Type Culture Collection,  
18 replicated in Hela cells, and purified by standard  
19 techniques (Macket, et al., DNA Cloning: A Practical  
20 Approach, "The Construction and Characterization of  
21 Vaccinia Virus Recombinants Expressing Foreign Genes",  
22 pp. 191-211, IRL Press, Oxford. 1985). Human tk 143  
23 cells were grown in Eagle's medium with 10% fetal bovine  
24 serum (FBS). CVI and BSCI cells were grown in Dulbecco's  
25 modified medium (DMEM) containing 10% FBS.

26 Formation of gpt recombinant virus. Recombinant  
27 viruses were prepared by standard procedures as described  
28 by Macket, et al., supra with the following  
29 modifications:  $5 \times 10^6$  CVI cells (confluent monolayers)  
30 were infected with 0.2 plaque forming units



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1 (PFU) of vaccinia virus per cell. Two hours after  
2 infection, 1 ml of a calcium DNA precipitate (consisting  
3 of 5  $\mu$ g of supercoiled plasmid DNA, 1  $\mu$ g of vacinia virus  
4 DNA, and 14  $\mu$ g of sheared herring sperm DNA) was added to  
5 the cells. After 15 minutes of incubation at room  
6 temperature, 9 ml of medium (DMEM, 8% FBS with penicillin  
7 and streptomycin) were added. The medium was changed  
8 after 4 h and the incubation was continued for another 36  
9 to 48 hours. Virus stocks were prepared by resuspending  
10 the infected cells in 1 ml of medium, freezing and  
11 thawing three times.

12 Selection of gpt virus. For the isolation of gpt  
13 recombinants, a plaque assay on BSCI cells was done as  
14 follows: confluent BSCI cells were preincubated in the  
15 gpt selection medium (DMEM, 2.5% FBS, 25  $\mu$ g/ml MPA, 250  
16  $\mu$ g/ml xanthine, and 15  $\mu$ g/ml hypoxanthine) for 14 to 24  
17 hours. The virus stock was digested with an equal volume  
18 of trypsin (0.25 mg/ml) for 30 minutes at 37°C and  
19 sonicated for 20 seconds on ice. Dilutions ( $10^{-3}$ ,  $10^{-4}$ ,  
20 and  $10^{-5}$ ) of the trypsinized virus stock were used to  
21 infect the BSCI cells. After 1.5 hours of incubation at  
22 37°C, the cells were overlaid with the gpt-selective  
23 medium containing 1% of low melting agarose. After 2  
24 days of incubation, the cells were stained with neutral  
25 red (Gibco). The plaques were readily visible after  
26 overnight incubation.

27 Preparation and analysis of DNA. Recombinant  
28 plasmids were constructed and isolated by standard  
29 methodologies as described by Maniatis, et al., Molecular  
30 Cloning, Cold Spring Harbor Labs, Cold Spring Harbor, New  
31 York, 1982. For the genomic analysis of recombinant  
32 viruses,  $2.5 \times 10^6$  BSCI cells were infected with the

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1 material obtained from a single plaque and grown for 24  
2 hours in selective medium. Total cellular DNA was  
3 extracted, digested with restriction endonuclease  
4 HindIII, electrophoresed through a 1% agarose gel, and  
5 subjected to Southern blot analysis following standard  
6 procedures.

7 Construction of recombinant plasmids. pTK61-gpt:  
8 HindIII linkers were added to the HindIII-HpaI fragment  
9 of *E. coli* gpt gene obtained from plasmid pSV2-gpt.  
10 Subsequently, the fragment was inserted into the unique  
11 HindIII site of pGS61, resulting in the plasmid  
12 pTK61-gpt.

13 pP11: The HindIII and SstI site, flanking the 11  
14 K gene promoter in plasmid pSC42, were converted into  
15 XhoI sites by T4 polymerase treatment and ligation of  
16 XhoI linkers. The plasmid pSC42 contains the ClaI-EcoRI  
17 11 K gene promoter fragment cloned into the SphI site of  
18 pUC19.

19 pTKgpt-F1s and pTKgpt-oF1s: The construction of  
20 these plasmids is outlined in detail in Figure 3.

21 pTKgpt-F2s and pTKgpt-F3s: The frameshift  
22 mutations were done by standard oligonucleotide directed  
23 mutagenesis. To obtain pTKgpt-F2s, a 30-mer  
24 oligonucleotide (5'-GACCTGCAGGAATTCATTATAGCATAGA-3'),  
25 and to obtain pTKgpt-F3s, another 30-mer  
26 (5'-ACCTGCAGGAATTCCTCA-  
27 TTTATAGCATAGA-3'), were used. Screening of the mutants  
28 was done by standard plasmid sequencing (Hattori, et al.,  
29 Anal. Biochem. 152:232-240, 1986) with the help of the  
30 20-mer primer (5'-GCGATGCTACGCTAGTCACA-3') derived from

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1 the upstream region of the 11 K gene promoter  
2 (nucleotides -96 to -76 upstream of the initiation  
3 codon). The primary structure around the 11 K promoter  
4 region and the unique cloning sites in all vectors were  
5 confirmed by standard plasmid sequencing.

6 ptKgpt-F1sB and pTKgpt-oF1sB: The BamHI fragment  
7 of pMC1871 containing the E. coli lacZ gene (Shapira, et  
8 al. 1983, Gene 25:71-82) was cloned into the BamHI sites  
9 of pTKgpt-F1s and pTKgpt-oF1s.

10 A deposit of pTKgpt-F1s, pTKgpt-F2s and pTKgpt-F3s  
11 has been made at the ATCC on March 18, 1988 under  
12 accession numbers 67,655, 67,656 and 67,657,  
13 respectively. The deposits shall be viably maintained,  
14 replacing if it became non-viable, for a period of 30  
15 years from the date of the deposit, or for 5 years from  
16 the last date of request for a sample of the deposit,  
17 whichever is longer, and made available to the public  
18 without restriction in accordance with the provisions of  
19 the law. The Commissioner of Patents and Trademarks,  
20 upon request, shall have access to the deposit.

21 pTKgpt-F2sB: The SmaI-SalI fragment of pMC1871  
22 was inserted into the HincII site of pTKgpt-F2s.

23 pTKgpt-F3sB: The SalI fragment of pMC1871 was  
24 cloned into the SalI site of pTKgpt-F3s.

25 Mycophenolic acid inhibition of the growth of  
26 vaccinia virus. The mycotoxin MPA inhibits the enzyme  
27 inosine monophosphate dehydrogenase and thereby prevents  
28 the formation of xanthine monophosphate. This results in  
29 the intracellular depletion of purine nucleotides and in

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1 an inhibition of cell growth. Treatment of host cells  
2 with MPA, therefore, severely inhibits the growth of  
3 viruses. This was established by testing the effect of  
4 increasing amounts of MPA on the plaque formation of  
5 vaccinia virus. It was found that 25  $\mu$ g/ml of MPA in the  
6 medium results in a nearly complete inhibition of plaque  
7 formation in all cell lines tested (BSCI, CVI, and human  
8 tk<sup>-</sup> 143 cells). In BSCI and CVI cells, only a few tiny  
9 plaques could be observed on crystal violet-stained  
10 monolayers after 2 days of incubation (Fig. 1A).  
11 Replacement of the selective medium with normal medium  
12 resulted in plaque formation comparable to the control  
13 (Fig. 1B), indicating that the inhibition is reversible.

14 Expression of the E. coli gpt gene and its effect  
15 on plaque formation in the presence of MPA. The  
16 inhibition of the de novo synthesis of purines by MPA can  
17 be overcome by a cell that expresses the E. coli gpt  
18 gene, which codes for the enzyme xanthine-guanine-  
19 phosphoribosyl-transferase (XGPRT), in the presence of a  
20 substrate for purine metabolism such as xanthine and  
21 hypoxanthine in the growth medium. To determine whether  
22 the block of purine synthesis by MPA can also be overcome  
23 by a recombinant virus expressing the bacterial XGPRT,  
24 first the plasmid pTK61-gpt was constructed. In this  
25 construct, the gpt gene is controlled by the promoter  
26 from the vaccinia virus 7.5 K gene and is flanked by  
27 viral tk sequences. The 7.5 K gene promoter was chosen  
28 because it is active early and late in infection and  
29 might provide continuous production of the bacterial  
30 purine salvage enzyme. The plasmid was transfected into  
31 CVI cells that were infected with wild-type virus so that  
32 the gpt gene would be recombined into the viral tk  
33 locus. Putative recombinants were detected by a plaque

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1 assay on BSCI cells in the presence of MPA, xanthine, and  
2 hypoxanthine as described herein. Large plaques formed  
3 only when the gpt gene was used for transfection,  
4 indicating that the desired recombinants behaved in the  
5 desired manner. One of the recombinants was plaque  
6 purified twice under selective conditions and a small  
7 virus stock was grown. A plaque assay demonstrated that  
8 this recombinant, in contrast to the wild type virus,  
9 formed plaques on BSCI cells in the presence of selective  
10 medium (Fig. 1C).

11 The genomic analysis of virus grown from six  
12 randomly picked plaques that formed during the first  
13 selection step is shown in Fig. 2. The HindIII fragments  
14 of all six genomes contain the anticipated 2.0 kbp  
15 fragment that hybridizes with the gpt probe (Fig. 2A).  
16 After washing off the labeled DNA, the same Southern  
17 filter was hybridized to a vaccinia virus tk gene  
18 specific probe (Fig. 2B). The tk sequences can be  
19 detected as a large fragment (4.7 kbp) and a small one  
20 (1.0 kbp), indicating the integration of the gpt gene  
21 into the viral tk locus. Since all plaques picked after  
22 the first selection step have integrated the selective  
23 marker, no other screening procedures are necessary to  
24 identify a viral recombinant. Thus the present invention  
25 provides a single step procedure for selection of  
26 dominant recombinants.

27 Construction of the insertion and expression  
28 vectors pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s. A series  
29 of plasmids was constructed that use the gpt gene as a  
30 selective marker and that allow the expression of foreign  
31 genes controlled by the promoter of the major late 11 K  
32 polypeptide (Fig. 3). The 5' regulatory region of the 11  
33 K gene lies within a 30 kbp segment located immediately

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1 upstream of the ATG initiation codon. Since the  
2 initiation codon forms part of a highly conserved TAAATG  
3 sequence within which the 5' ends of late mRNAs map, this  
4 region was chosen not be altered. The presence of an  
5 EcoRI site immediately downstream of the ATG facilitated  
6 the insertion of a polylinker with multiple unique  
7 cloning sites. Three vectors in which 0, 1, or 2  
8 guanosine residues follow the ATG allow any coding  
9 sequences to be inserted in the correct reading frame.  
10 These vectors also provide all-frame stop codons at the  
11 end of the polylinker. The sequences downstream of the  
12 11 K gene initiation codon for the three vectors (termed  
13 pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s) are shown in Fig.  
14 4. The vector pTKgpt-oF1s (Fig. 3) is the orientation  
15 isomer to pTKgpt-F1s and has, therefore, the same  
16 sequence downstream of the 11 K gene ATG as pTKgpt-F1s.

17 Formation of recombinant vaccinia viruses that  
18 express  $\beta$ -galactosidase. To ensure the normal  
19 functioning of the vector constructs and to be able to  
20 easily quantitate the amounts of protein expressed, E.  
21 coli lacZ gene fragments that lack their own initiation  
22 codons (but still have their own termination codons) were  
23 inserted in frame into an appropriate restriction site of  
24 each of the four vectors. Four plasmids were obtained  
25 that were termed pTKgpt-F1sB, pTKgpt-F2sB, pTKgpt-F3sB,  
26 and pTKgpt-oF1sB. These plasmids were employed to  
27 construct viral recombinants. In each case, all the  
28 plaques obtained under selective conditions were able to  
29 convert the XGal in an agar overlay into its blue  
30 hydrolysis product. This indicated the co-expression of  
31 the selected marker gene and the gene of interest and  
32 shows that the lacZ gene fragments were in the predicted  
33 reading frames.

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To quantitate the amounts of  $\beta$ -galactosidase produced by the viral recombinants, two gpt<sup>+</sup> viruses, derived from the vectors pTKgpt-F1s and pTKgpt-oF1s, were plaque purified three times and small stocks were grown in CV1 cells. These stocks were used to infect CV1 cells at a multiplicity of 7.5 PFU of the respective virus. For comparison, the same assay was done also with a virus (vtat) based on the vector pSC11 that also expressed the lacZ gene driven by the 11 K gene promoter (but the  $\beta$ -galactosidase of which has a slightly different N-terminus), and with a vaccinia virus-T7 RNA polymerase hybrid system that expresses the lacZ gene behind the phage T7 promoter after co-infection with a T7 polymerase producing virus. The results of this analysis are shown in Fig. 5. The viruses based on the vectors pTKgpt-F1s, pTKgpt-oF1s, and pSC11 express similar amounts of  $\beta$ -galactosidase, indicating that lacZ gene activity is relatively independent of the orientation and of the kind of neighboring sequences in the virus. The specific activity of pure  $\beta$ -galactosidase is 300,000 units/mg. Based on this number, the bacterial enzyme produced by virus-infected cells is more than 3% of the total cellular protein, an amount that is easily detectable in a Coomassie blue stained gel. In fact, a strong band in the 100,000 kDa range was observed upon electrophoresis of proteins from cells infected with a pTKgpt-F1s based virus, and not in the proteins of wild-type virus infected cells. The level of  $\beta$ -galactosidase expression using the 11 K promoter was about 2-fold higher than that achieved with the hybrid vaccinia virus-T7 RNA polymerase system under the infection conditions described in Fig. 5.

In summary, in accordance with the present invention, the gpt gene is incorporated into a plasmid

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1 vector that has a vaccinia promoter and unique  
2 restriction endonuclease sites for insertion of a foreign  
3 gene. Because of vaccinia derived flanking sequences,  
4 the entire selection-expression cassette is inserted as a  
5 unit into the vaccinia virus genome by homologous  
6 recombination. Thus, all of the gpt<sup>+</sup> recombinants  
7 analyzed also contain the foreign gene that has been  
8 inserted into the plasmid vector. For convenience, the  
9 flanking sequence used in this study were derived from  
10 the vaccinia tk gene; however, since tk selection is no  
11 longer required in accordance with the method of the  
12 present invention, any non-essential site in the vaccinia  
13 genome can be employed.

14 In order to achieve high levels of expression, the  
15 promoter chosen for the vectors was derived from the  
16 major 11 k structural protein. However, other promoters  
17 well known to one of ordinary skill in the art could also  
18 be used. Although the mechanism of late transcription is  
19 still poorly understood and involves the attachment of a  
20 unique 5' poly(A) leader, the important sequences are  
21 contained within a relatively small region starting about  
22 30 bp upstream of the RNA start site and include the  
23 translation initiation codon which is a part of the  
24 conserved TAAATG sequence. For this reason, the  
25 insertion sites for foreign genes were placed just  
26 downstream of the ATG. Since the vectors may prove  
27 useful for expressing open-reading-frames, multiple  
28 cloning sites were engineered in all three frames as well  
29 as termination codons. The efficacy of the system was  
30 illustrated by expression of  $\beta$ -galactosidase; the yield  
31 of enzyme was found to be greater than about 3% of the  
32 total cell protein. This expression level is higher than  
33 that obtained using the more widely used 7.5 K promoter  
34 and exceeded even that obtained with the recent



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1 bacteriophage T7 vaccinia hybrid system (Fuerst, et al.,  
2 Mol. Cell. Biol., 7:2538-2544, 1987). The vectors  
3 described herein could, of course, also be employed for  
4 direct cloning and expression of open-reading-frames in  
5 mammalian cells as well, in a manner similar to that used  
6 routinely with bacteriophage  $\lambda$  in *E. coli*.

7 Clearly, the gpt selection provides a number of  
8 important advantages over previous procedures devised to  
9 isolate recombinant vaccinia viruses. These include  
10 one-step plaque isolation without need for enrichment,  
11 application to a variety of cell lines, use of  
12 alternative insertion sites in the vaccinia genome, and  
13 absence of spontaneous selectable mutants. In addition,  
14 5-bromodeoxyuridine, which is used for tk selection, is  
15 highly mutagenic, whereas mycophenolic acid is  
16 non-mutagenic in the Ames test and in the related SOS  
17 test. Avoidance of mutagens ensures virus stability. It  
18 is noted that gpt selection method could also be used  
19 with other virus vectors, including other members of the  
20 poxvirus family, herpesviruses, adenoviruses,  
21 retroviruses, and baculoviruses.

22 It is understood that the examples and embodiments  
23 described herein are for illustrative purposes only and  
24 that various modifications or changes in light thereof  
25 will be suggested to persons skilled in the art and are  
26 to be included within the spirit and purview of this  
27 application and scope of the appended claims.

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1     WHAT IS CLAIMED IS

2           1.    An    expression    vector,    comprising a  
3    recombinant vaccinia virus in genome of which is  
4    incorporated an E. coli gpt gene and one or more foreign  
5    genes desired to be expressed by the recombinant virus,  
6    said recombinant virus forming plaques on a plurality of  
7    cell lines when replicated in a growth medium comprising  
8    sufficient amount of mycophenolic acid to inhibit purine  
9    metabolism in the presence of sufficient amount of an  
10   unphosphorylated purine substrate.

11           2.    The expression vector of claim 1 further  
12   comprising a strong late promoter flanked by nonessential  
13   vaccinia sequences and multiple restriction sites in  
14   different open frames for expression of partial or  
15   complete foreign genes.

16           3.    A method for selecting recombinant vaccinia  
17   vectors, comprising allowing recombinant vaccinia vectors  
18   to replicate on an infectable cell line in a growth  
19   medium comprising sufficient amount of mycophenolic acid  
20   to inhibit purine metabolism in the presence of  
21   sufficient amount of an unphosphorylated purine substrate  
22   and then isolating plaque forming recombinants therefrom  
23   by convention techniques.

24           4.    The method of claim 3 wherein said purine  
25   substrate is selected from the group consisting of  
26   xanthine, hypoxanthine and combination thereof.

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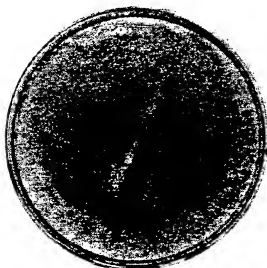
1           5.    A plasmid comprising vaccinia virus DNA  
2    containing an E. coli gpt gene controlled by vaccinia  
3    virus promoter and a second vaccinia virus promoter next  
4    to a restriction endonuclease site for insertion of a  
5    foreign gene.

6           6.    The plasmid of claim 5 having the  
7    identifying characteristics of ATCC 67,655.

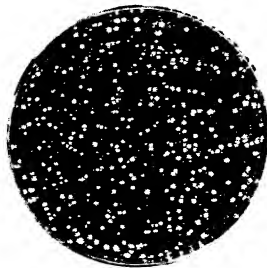
8           7.    The plasmid of claim 5 having the  
9    identifying characteristics of ATCC 67,656.

10          8.    The plasmid of claim 5 having the  
11   identifying characteristics of ATCC 67,657.

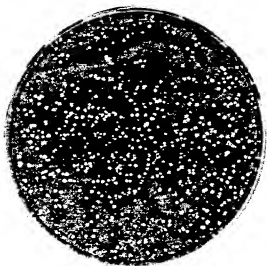
**FIG. 1A**



**FIG. 1B**



**FIG. 1C**



**FIG. 1D**

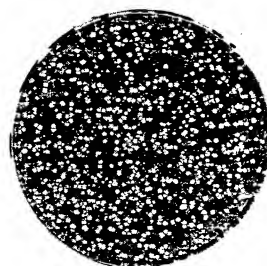
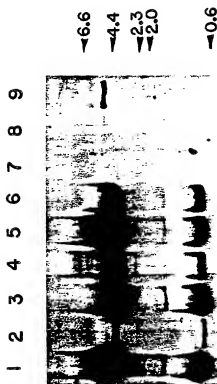


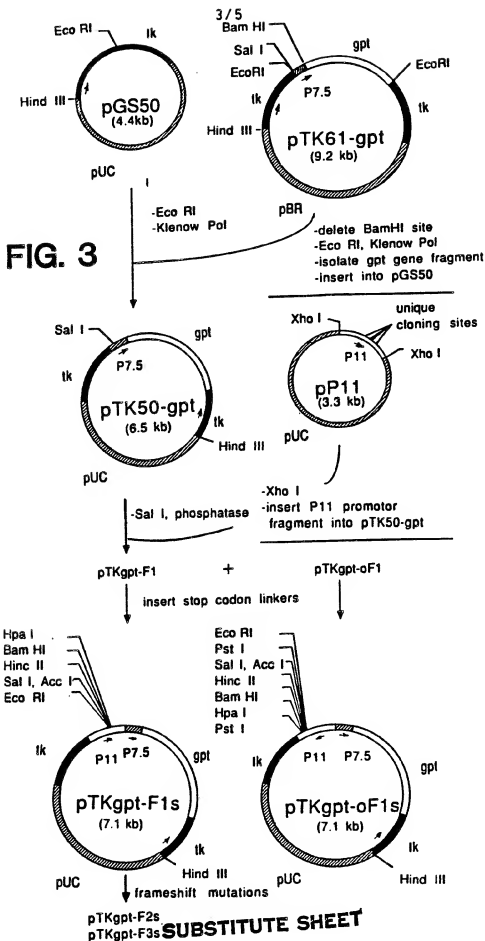
FIG. 2A



FIG. 2B



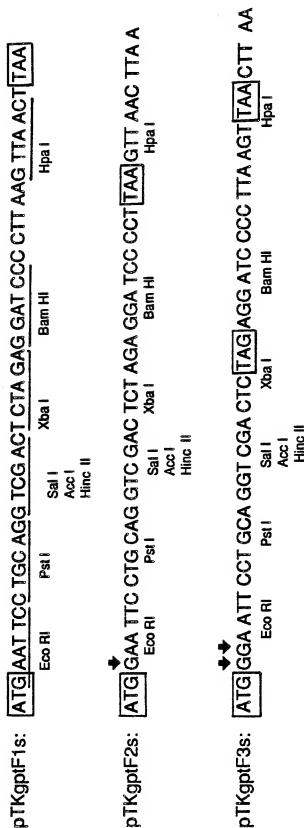
2/5



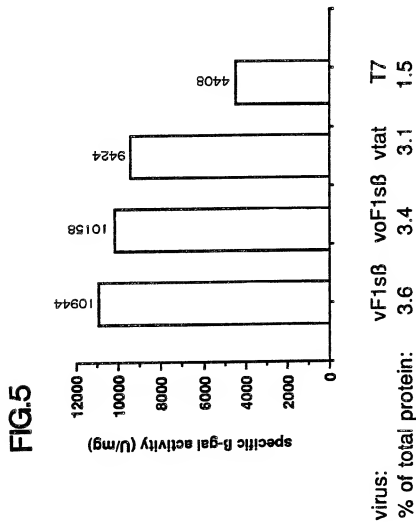
**SUBSTITUTE SHEET**

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FIG.4



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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00931

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(4): C12Q 1/48, 1/70; C12N 7/00, 15/00

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
U.S.	435/5, 15, 172.3, 320

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched \*

CAS FILE 1967-1989  
 BIOSIS FILE 1967-1989

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y, P	Gene, Volume 65, No. 1, published 15 May 1988. D.B. Boyle, et al. "A dominant selectable marker for the construction of recombinant poxviruses," pp. 123-128. See entire document.	1-8
X, P	Journal of Virology, Volume 62, No. 6, published June 1988. F.G. Falkner, et al. " <u>Escherichia coli</u> gpt gene provides dominant selection for vaccinia virus open reading fram expression vectors," pp. 1849-1854. See entire document.	1-8
Y	Plasmid, Volume 18, No. 1, published July 1987. B. Shepard, et al. "Transient expression system to measure the efficiency of vaccinia promoter regions", pp. 16-23, see Abstract and Results Section.	1-8

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 JUNE 1989

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

23 JUN 1989

Signature of Authorized Officer

BETH A. BURROUS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Science, Volume 232, published 27 June 1986/ G. Thomas, et al. "Expression and cell type-specific processing of human proenkephalin with a vaccinia recombinant," pp. 1641-1643. See entire document.	1,2 5-8
Y	Proc. Natl. Acad. Sci. USA, Volume 78, No. 4, published April 1981. R.C. Mulligan, et al. "Selection for animal cells that express the <i>Escherichia coli</i> gene coding for xanthine-guanine phosphoribosyltransferase," pp. 2702-2706. See entire document.	1,3-8
Y	Proc. Natl. Acad. Sci. USA, Volume 82, No. 7, published April 1985. C. Bertholet, et al. "One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription", pp. 2096-2100. See abstract, Figure 2 and Results Section.	1-8
Y	Molecular and Cellular Biology, Volume 5, No. 8, published August 1985. C.A. Franke, et al. "Neomycine resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants", pp. 1918-1924. See entire document.	1-8